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Breast cancer has a high rate of mortality. The ability to monitor micrometastatic breast cancer cells via their protease production may aid the clinician in managing the disease. Specifically, we have proposed that elevated plasma protease (MMP-9, MMP-2 and heparanase) levels are indicative of tumor progression. Further, if this increased protease(s) can be detected prior to the onset of clinical manifestations of progressive disease (e.g. lytic bone disease, rising CEA) then patients can be rapidly changed over to an alternative therapeutic modality. Towards this end, we have utilized a fluorescence immunosorbent assay to measure the amount of these plasma enzymes. In preliminary studies, we have found that during treatments with either vinblastine, methotrexate/vinblastine or taxol, plasma MMP-9 levels increase in those patients who do not respond to these regimens. The increase in this type IV collagenase is unlikely to reflect variations in the white blood count since we found a poor relationship between the amount of this enzyme and absolute neutrophil count. We are currently expanding these studies to include MMP-2 and heparanase and to determine if rising MMP-9 levels is always associated with treatment failure to the abovementioned regimens or restricted to a subset of patients.

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INTRODUCTION

Breast cancer in women carries a high rate of mortality this reflecting in part the spread of this disease to other organ sites a process referred to as metastasis. While the dissemination of breast cancer is a highly complex process, it is widely accepted that the destruction of the surrounding tissue including the extracellular matrix is a strict requirement. This is achieved via the expression of multiple proteases either by the tumor cells or by the neighboring cells such as fibroblasts or infiltrating macrophages recruited by the malignant cells (1-4). Three of the proteases which have been implicated in breast cancer spread are the MMP-9 (also referred to as gelatinase B) and MMP-2 (also referred to as gelatinase A) type IV collagenases as well as heparanse (5,6).

The MMP-9 gene is comprised of 13 exons stretching over 7.7 kb, and is located on chromosome 20 (7). Zucker and co-workers reported that this collagenase was increased in patients with either breast and colon cancer (5). Similarly, Nakajima and others found that serum and plasma MMP-9 levels correlated with spontaneous metastasis of rat 13762NF mammary adenocarcinoma (8). The primary source of the MMP-9 is probably the carcinoma cells with minor contributions from the neighboring stromal fibroblasts and endothelial cells as shown by *in situ* hybridization (6).

MMP-2 is, like MMP-9, a type IV collagenase. However, this protein is encoded on a separate gene located on chromosome 16 (9) and its expression is regulated quite separately to that of gelatinase B (10). The main substrate of MMP-2 is also basement membrane type IV collagen (11) although it may also play a role in the activation of MMP-9 (12). Recently, it was shown that the cleavage of laminin-5 by this collagenase resulted in the migration of breast epithelial cells (13).The contribution of this collagenase to invasion and in some instances the metastasis of a divergent set of malignancies has been reported by several groups (14,15).

Heparanase is an endoglycosidase that targets heparan sulfates in the extracellular matrix (16-18). Previous studies have shown that this enzyme is released from metastatic tumor cells and circulates in the body fluids of tumor-bearing rodents. For example, the amount of this enzyme steadily rose in the serum of female F344 rats injected into the fat pads with highly metastatic mammary adenocarcinoma (16). On the other hand, the level of this enzyme was low in rats injected with tumor cells with low metastatic potential.

Since these collagenases and heparanase have been implicated in tumor progression as shown by this group for breast cancer (19), we have hypothesized that these enzymes may be of utility in determining the response of breast cancer patients to current therapeutic strategies. Specifically, if the rising amount of one or more of these proteases is indicative of treatment failure, then other therapeutic options can be implemented. Such treatment decisions are currently based on standard clinical criteria such as computed tomography, bone scans, rising CEA and alkaline phosphatase as well as subjective criteria such as bone pain. If, however, our data show that a rise in plasma protease levels precedes the manifestation of clinical indices prognostic of tumor progression, this may allow for a faster switch over to other therapeutic agents/modalities. This in turn could yield more effective treatments of breast cancer patients culminating in increased survival times.

BODY

Experimental Methods

To determine if protease amounts were predictive of patient response, serial plasma samples from breast cancer patients were assayed for MMP-9 levels using a fluorescence immunosorbent assay.

Results and Discussion

Patient # 1 was a 59 year old female diagnosed with Blacks nuclear grade I, lymph node-positive breast cancer in November of 1992. The patient, was treated with combined 5-fluorouracil/adriamycin/cytoxan (FAC) after surgery and this treatment phase was associated with a decline in MMP-9 levels to below that of the average of normal persons (110 ng/ml determined from 10 healthy individuals from whom at least 3 plasma collections were made). At the end of June 1993, the patient completed the treatment course and was switched over to combined methotrexate/vinblastine. Interestingly, for the duration of this treatment, plasma MMP-9 levels continued to rise to a maximum concentration of 470 ng/ml. However, after a 2 month treatment period, this patient was deemed asymptomatic based on clinical criteria and had completed the methotrexate/vinblastine treatment. The increase in the amount of this collagenase observed during methotrexate/vinblastine treatment therefore may have been indicative of tumor progression in contrary to clinical findings. At this point the patient was put on tamoxifen and in response to the anti-estrogen, the plasma concentration of MMP-9 diminished over 66 %. However, a rising CEA

level and a chest CT scan confirmed tumor progression in June of 1994. Thus, for this patient at least, it does not appear that plasma MMP-9 is indicative of tumor progression while on tamoxifen. The patient expired in December of that year (1994).

Patient # 2 was a 57 year old woman diagnosed with invasive ductal breast cancer. A mastectomy was performed in August of 1992 and this indicated a lymph node-positive tumor of 4.3 cm in size. The patient was treated in September of 1993 with taxol. However, for the 4 month duration of treatment, MMP-9 levels steadily rose from a pretreatment level of about 100 ng/ml to a peak amount of 500 ng/ml. At this point, the patient was deemed by standard clinical criteria (skull lytic lesions) to be progressing and thus switched over to tamoxifen. Like patient # 1, MMP-9 concentrations rapidly decreased to a level comparable to that of normal control patients (100 ng/ml). This level was maintained for the treatment duration although the disease was judged as progressing based on a bone scan. However, cessation of the anti-estrogen was accompanied by a sharp increase in the amount of this collagenase (790 ng/ml) in the plasma. Treatment with aminoglutethemide, which inhibits estrogen production, was associated with a return of MMP-9 levels to that of normal controls. Again, cessation of treatment, due to disease progression, was associated with a rise in the amount of this metalloproteinase. The patient expired in May 1995. These data are consistent with the notion that plasma MMP-9 levels are indicative of tumor progression at least in taxol-treated breast cancer patients.

Patient # 3 was a 55 year old female diagnosed with invasive, poorly differentiated breast cancer. The patient was placed on Megace (megestrol acetate) and this was accompanied by an 85 % reduction in MMP-9 levels achieved in July of 1993. However, at the end of the treatment period, there was a sharp spike (600 ng/ml) in the level of this collagenase (8/12/93) which transiently decreased upon tamoxifen therapy. The patient was deemed as having tumor progression and was switched over to cytoxan/methotrexate/5-Flurouracil (CMF) (9/30/93) and taxol thereafter (12/9/93). A steady increase in MMP-9 levels was evident under these regimens and indeed this patient was assessed as having tumor progression (based on a bone scan) and accordingly, the taxol replaced with vinblastine (3/21/94). This change in chemotherapeutic agent initially caused an MMP-9 reduction but subsequently the amount of MMP-9 in the plasma increased two-fold and in mid June 1994, chemotherapy was stopped as a consequence of treatment failure (extensive lytic disease within tibia and pelvis). The patient was thus changed over to hormonal intervention (Halotestin-androgen). MMP-9 levels subsequently decreased by over 50 % but started to increase shortly thereafter (September 1994). The patient expired in September 1995.

To confirm that the plasma of the patients contained MMP-9, various dilutions of samples from patients # 1 (12/7/92) and # 2 (11/4/93) were subjected to zymography (Figure 4). In these assays, samples are subjected to SDS-PAGE in a gel containing gelatin. SDS is removed from the gel by extensive washing. The gel is subsequently incubated at 37 °C to allow gelatinolysis to proceed. Subsequently, the gel is stained with Coomassie and collagenase activity is detected as a white band in a dark background. A band which co-migrated with the 92 kDa molecular weight standard was observed in plasma from both patients. Moreover, the upper band was indistinguishable in size to that of MMP-9 obtained from a positive control cell line (UM-SCC-1). Note also the presence of a lower band which was indistinguishable in size from the 72 kDa type IV collagenase (MMP-2). Thus, these data indicate the presence of both of these type IV collagenases in the plasma of breast cancer patients.

It should be emphasized that the elevation in MMP-9 levels in the plasma of the breast cancer patients is unlikely to reflect fluctuations in the absolute neutrophil count (see Table below).

Recommendations in relation to Statement of Work

Clearly, the results are preliminary. We will continue to assay the plasma samples from a greater number of breast cancer patients for MMP-9 to determine if the abovementioned trend is representative of all patients or of a sub-population. Additionally, the utility of MMP-2 and heparanase either, individually or in combination, with the other 2 proteases, in predicting treatment failure will be determined.
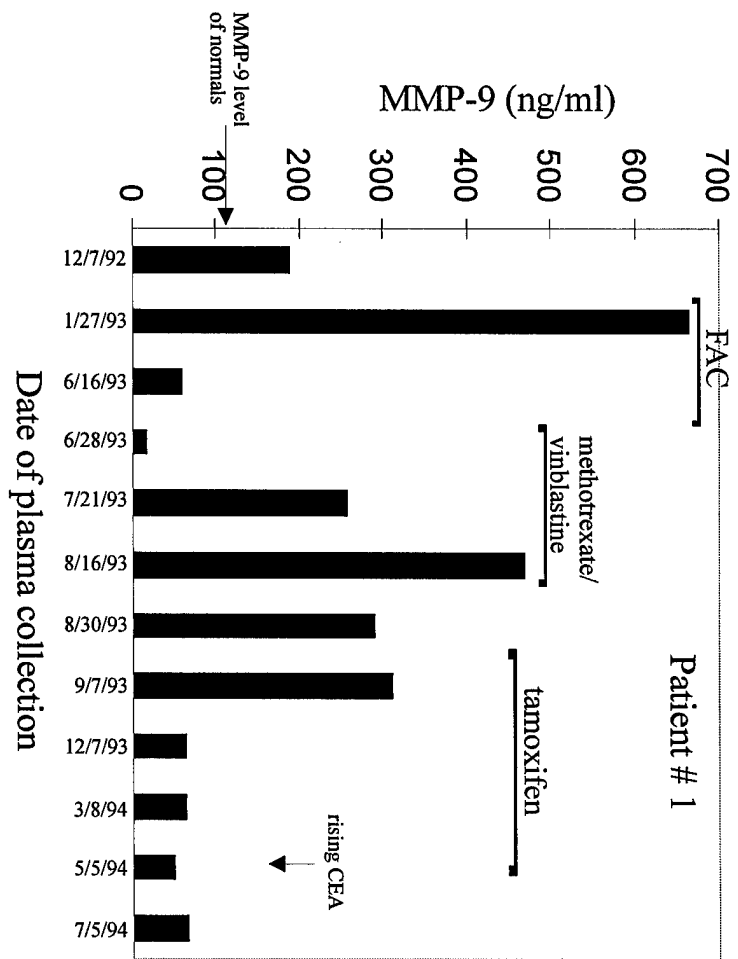
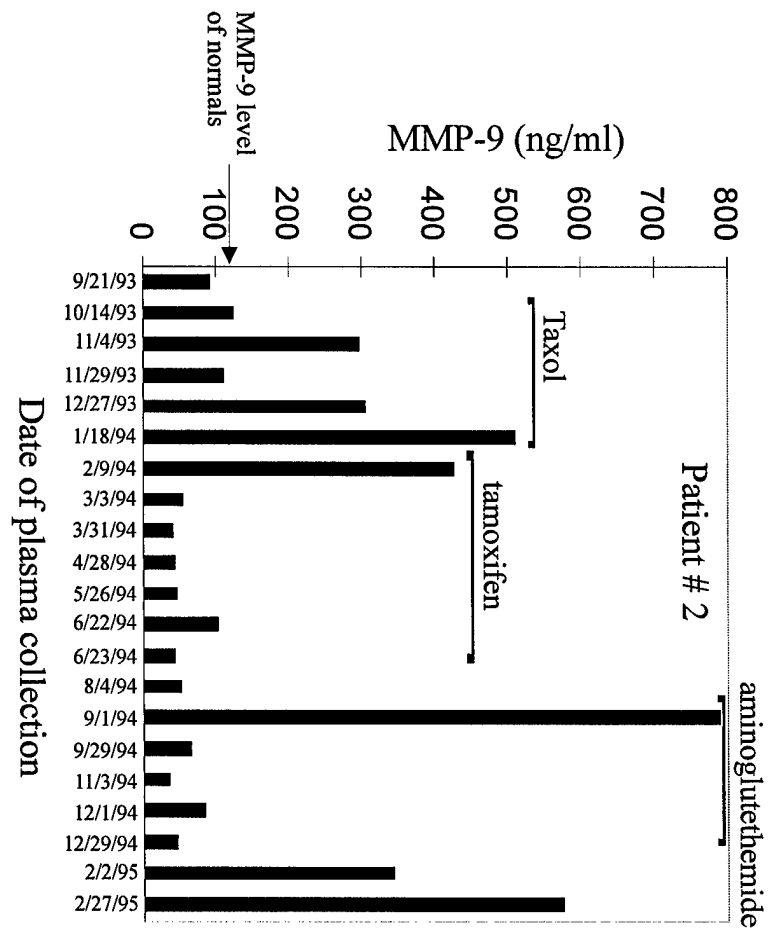| Patient #	MMP-9 (ng/ml)	Absolute Neutrophil Count (k/ul)
2	51	5.39
2	788	6.8
2	344	9.9
3	131	3.7

6

3	234	3.5
3	364	2.6
3	327	4.4

CONCLUSIONS

Although these findings are based on a small patient set, it is tempting to speculate that a rise in plasma MMP-9 levels is indicative of treatment failure with taxol, vinblastine and combined methotrexate/vinblastine. Further and equally important, the data suggest that the increase in MMP-9 amounts can, at least in some cases, be detected prior to the manifestation of clinical criteria used to judge treatment efficacy.

However, with the current set of data, we did not observe a trend with patients undergoing hormonal manipulation (tamoxifen, halotestin, aminoglutethemide). Thus, while these treatments decreased plasma MMP-9 amounts, a rise in this protease was not apparent when the disease was progressing as determined by standard clinical criteria. It is not presently clear as to why tumor progression can be detected in patients treated with chemotherapeutic agents but not with hormonal drugs.
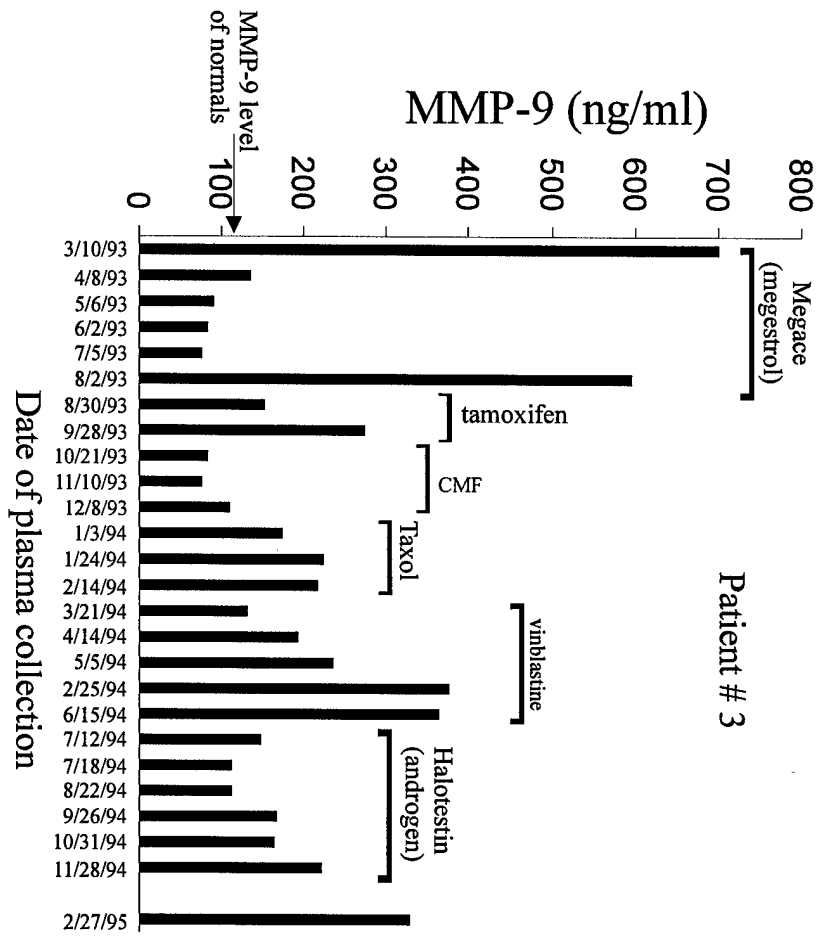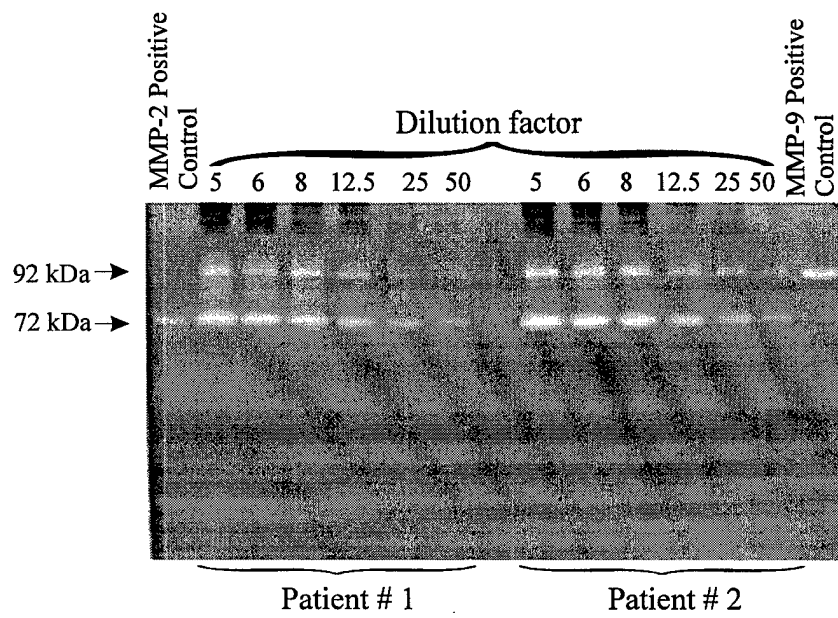
MMP-9 (ng/ml)

MMP-9 level of normals

FAC

methotrexate/ vinblastine

tamoxifen

rising CEA

Patient # 1

Date of plasma collection

MMP-9 (ng/ml)

0   100   200   300   400   500   600   700   800

MMP-9 level
of normals

Date of plasma collection

3/10/93
4/8/93
5/6/93
6/2/93
7/5/93
8/2/93
8/30/93
9/28/93
10/21/93
11/10/93
12/8/93
1/3/94
1/24/94
2/14/94
3/21/94
4/14/94
5/5/94
2/25/94
6/15/94
7/12/94
7/18/94
8/22/94
9/26/94
10/31/94
11/28/94
2/27/95

Megace
(megestrol)

tamoxifen

CMF

Taxol

vinblastine

Halotestin
(androgen)

Patient # 3

Figure 4

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APPENDICES

NONE